North Wind

Poultry Quantitative PCR Analytical Summary

January 21, 2008

Overview:

The objective of this project was to quantify the number of poultry-specific *Brevibacteria* biomarker gene copies contained in water, soil, and/or litter samples using quantitative polymerase chain reaction (qPCR). The client is Camp, Dresser and McKee. Table 1 describes the sample matrix and the condition of the samples upon arrival to the analytical laboratory.

Table 1. Description of samples and volume or mass filtered for DNA extraction.

Sample ID	Matrix/ Date Sampled	Condition Received/Observations	Volume Filtered (mL) or Mass Extracted (g)
16861-7-20-06	Water/7-20-06	Cold/bottle intact	250
BLFOSP-6-7-06	Water/6-7-06	Cold/bottle intact	500
BS-HF22A-5-2-07	Water/5-2-07	Cold/bottle intact	500
BS-HF22-SW-8-24-05	Water/8-24-05	Cold/bottle intact	500
ELMSPRQ-6-28-06	Water/6-28-06	Cold/bottle intact	500
EOF-07-230-4-24-07	Water/4-24-07	Cold/bottle intact	200
EOF-07-232-4-24-07	Water/4-24-07	Cold/bottle intact	100
EOF-259-4-13-07	Water/4-13-07	Cold/bottle intact	20
EOF9-6-8-05	Water/6-8-15	Cold/bottle intact	500
EOF-Q1-6-17-06	Water/6-17-06	Cold/bottle intact	50
EOF-Q4-6-18-06	Water/6-18-06	Cold/bottle intact	100
EOF-SPRD-26-4-25-06	Water/4-25-06	Cold/bottle intact	100
EOF-SPREAD-025-5-4-06	Water/5-4-06	Cold/bottle intact	100
EOF-SPREAD-26-01-4-29-06	Water/4-29-06	Cold/bottle intact	100
EOF-SPREAD-59-01-4-29-06	Water/4-29-06	Cold/bottle intact	100
GPGW-20-6-11-30-06	Water/11-30-06	Cold/bottle intact	50
GPGW-48-7-12-1-06	Water/12-1-06	Cold/bottle intact	50
GW-Choats-01	Water	Cold/bottle intact	1000
GW-JONES-012311	Water	Cold/bottle intact	500
GW-McCOY-012212	Water	Cold/bottle intact	500
HESTENSP1-6-8-06	Water/6-8-06	Cold/bottle intact	500
HFS-02-5-10-06	Water/5-10-06	Cold/bottle intact	200
HFS-02-INITIAL-4-29-06	Water/4-29-06	Cold/bottle intact	400
HFS02Libby-6-15-05	Water/6-15-05	Cold/bottle intact	500
HFS04-BF1-01-6-15-06	Water/6-15-05	Cold/bottle intact	400
HFS05A-9-13-05	Water/9-13-05	Cold/bottle intact, label missing, sample ID assumed from lab and field note description	250
HFS05-BF1-01-6-15-06	Water/6-15-06	Cold/bottle intact	400
HFS05-BF1-7-12-05	Water/7-12-05	Cold/bottle intact	550
HFS14-BF2--01-8-1-06	Water/8-1-06	Cold/bottle intact	250
HFS-14PEAK-4-7-06	Water/4-7-06	Cold/bottle intact	250
HFS16-8-14-05	Water/8-14-06	Cold/bottle intact	300
HFS16-9-15-05	Water/9-15-06	Cold/bottle intact	300
HFS16-BF1-7-12-05	Water/7-12-05	Cold/bottle intact	300
HFS16-BF2-03-8-27-05	Water/8-27-05	Cold/bottle intact	250
HFS20-BF2--01-8-1-06	Water/8-1-06	Cold/bottle intact	250

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Sample ID	Matrix/ Date Sampled	Condition Received/Observations	Volume Filtered (mL) or Mass Extracted (g)
HFS22-9-15-05	Water/9-15-05	Cold/bottle intact, label missing, sample ID assumed from lab and field note description	250
HFS22A-6-15-05	Water/6-15-05	Cold/bottle intact	500
HFS22-BF1-01-6-15-06	Water/6-15-05	Cold/bottle intact	400
HFS22-BF2--01-8-1-06	Water/8-1-06	Cold/bottle intact	300
HFS23-7-16-05	Water/7-16-05	Cold/bottle intact	250
HFS23-7-23-05	Water/7-23-05	Cold/bottle intact	500
HFS-28A-5-10-06	Water/5-10-06	Cold/bottle intact	150
HFS-29PEAK-4-7-06	Water/4-7-06	Cold/bottle intact	250
LAL15SP2-7-11-06	Water/7-11-06	Cold/bottle intact	250
LAL16-GW1-7-18-06	Water/7-18-06	Cold/bottle intact	250
LIWSPR-6-28-06	Water/6-28-06	Cold/bottle intact	150
LK-04-0-01-5-16-06	Water/5-16-06	Cold/bottle intact	500
LK04-0-01-9-26-06	Water/9-26-06	Cold/bottle intact	250
LOC-01-4-7-06	Water/4-7-06	Cold/bottle intact	250
Osborn-7-20-06	Water/7-20-06	Cold/bottle intact	250
RS-03-01-7-12-06	Water/7-12-06	Cold/bottle intact	350
RS-109A-5-2-07	Water/5-2-07	Cold/bottle intact	500
RS-122-050307A	Water/5-3-07	Cold/bottle intact	500
RS-127-BIO-8-11-06	Water/8-11-06	Cold/bottle intact	350
RS-233-5-21-07	Water/5-21-07	Cold/bottle intact	250
RS-3-01-5-15-06	Water/5-15-06	Cold/bottle intact	500
RS-3-01-8-24-05	Water/8-24-05	Cold/bottle intact	500
RS-31-BIO-8-16-06	Water/8-16-06	Cold/bottle intact	250
RS-386A-5-2-07	Water/5-2-07	Cold/bottle intact	500
RS-399A-5-2-07	Water/5-2-07	Cold/bottle intact	250
RS-433A-5-2-07	Water/5-2-07	Cold/bottle intact	500
RS-574-BIO-8-10-06	Water/8-10-06	Cold/bottle intact	200
RS-577-BIO-8-11-06	Water/8-11-06	Cold/bottle intact	350
RS-578A-5-2-07	Water/5-2-07	Cold/bottle intact	500
RS-625-BIO-8-10-06	Water/8-10-06	Cold/bottle intact	500
RS-630-BIO-8-11-06	Water/8-11-06	Cold/bottle intact	250
RS-902-5-1-07	Water/5-1-07	Cold/bottle intact	500
RS-BALLARD-5-5-06	Water/5-5-06	Cold/bottle intact	150
SP-JONES-012307	Water/12-3-07	Cold/bottle intact	500
Spread-52-4-25-06	Water/4-25-06	Cold/bottle intact	100
ZPEOF-001-4-25-06	Water/4-25-06	Cold/bottle intact	100
ZPEOF-30-4-25-06	Water/4-25-06	Cold/bottle intact	150
HFS08A-6-15-05	Water/6-15-05	Cold/bottle intact	500
RS-122A-5-3-07	Water/5-3-07	Cold/bottle intact	500
Marth Guinn-7-25-06	Water/7-25-06	Cold/bottle intact	250
RS-3-01-9-25-06	Water/9-25-06	Cold/bottle intact	250
RS-340-BIO-8-15-06	Water/8-15-06	Cold/bottle intact	250
RS-43-BIO-8-10-06	Water/8-10-06	Cold/bottle intact	400

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The samples arrived in good condition at 4°C. All samples were received within 24 hours of sample collection. Upon arrival, the samples were filtered and frozen for storage at -80°C until the DNA extraction was performed. Following DNA extraction, the samples were first subjected to polymerase chain reaction (PCR) using universal bacterial probes in order to verify amplifiable DNA was present in the sample. In addition, for the 16S rRNA gene, a "nested" qPCR approach can be applied in which the universal bacterial PCR-amplified DNA is used as the template in a qPCR reaction. Although the results from the nested qPCR cannot be quantified per se, they can be used to lower the detect limit for the qPCR in order to determine if the poultry-specific *Brevibacteria* biomarker gene is present at concentrations lower than the method detect limit (MDL) using the groundwater DNA extractions. The results of these studies are described here.

Methods:

DNA Extraction. For soil and/or litter samples, DNA was extracted from 0.25 g of soil or litter using the FastDNA®SPIN® Kit for soil protocol. For surface water shipped to the laboratory, between 100 and 1,000 mL of groundwater was filtered through a Supor-200, 0.2 µm filter. The filters were frozen at -80°C and then shattered. Next, each sample tube was amended with 2 mL of DNA-free water, vortexed vigorously for 15 minutes, and the liquid volume was partitioned into DNA extraction tubes. DNA extractions were performed using the FastDNA®SPIN® Kit for soil according to the manufacturer's instructions. All DNA extractions were cleaned using an ethanol precipitation method. Community DNA was eluted in nuclease-free water (50 µL) and stored at -20°C.

Amplification of Bacteria. The PCR was used to amplify nearly full-length 16S rDNA genes from *Bacteria*. Each 25-µL PCR reaction included 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.5 µM each 8F forward and 907R reverse primer, 1 u/50uL Taq DNA polymerase, 0.2 mM dNTP, 1 µL template DNA, and 20 µL molecular-grade water. Amplification was performed on a MJ Research Peltier Gradient thermocycler using the following regime: 94°C (5 min) followed by 30 cycles of 94°C (1 min), 53.5°C (1 min), and 72°C (1 min, 50 sec). The reaction was finished with an additional 7 minutes at 72°C. PCR products were examined by ultraviolet (UV) light in a 1% agarose gel stained with ethidium bromide to confirm specificity of the amplification reactions.

Sepharose Cleanup. Any sample not amplifying in the PCR was processed through a Sepharose CL-4B (Sigma-Aldrich) size exclusion gel chromatography cleanup. Briefly, the micro-bio spin columns (Bio-Rad) were packed with sterile Sepharose CL-4B and washed with Tris-HCl buffer (pH 8). The sample was added to the packed gel column and eluted by spinning in a micro-centrifuge.

Detection of a Poultry Specific Brevibacteria Biomarker. The qPCR methods for assessing the 16S rRNA gene are very sensitive in detecting specific DNA fragments. The detection limit for the methods used is approximately 6 gene copies per µL of the DNA extraction. Biomarker DNA was cloned into a plasmid and was used as the source of the quantitative standards used in the analysis. Plasmid DNA containing the target 16S rRNA gene from the poultry-specific *Brevibacteria* biomarker was purified and quantified fluorometrically. Based on the known size of the plasmid and insert, DNA concentrations were converted to insert copy numbers. A dilution series spanning seven orders of magnitude was generated using known concentrations of each plasmid. Amplification and detection of the DNA was performed using the MJ Chromo-4 System. The acceptance criterion for the standard curve is a linear R^2 value of greater than 0.995.

To determine qPCR results, sample DNA diluted to a final concentration of 15 ng/5 µL DNA was combined with following reagents to reach a final concentration of 1X SYBR Green Master Mix and 0.5 µM 157F and 727R primer and water to reach 20 µL and 5 µL, respectively, of diluted sample DNA. Amplification was performed on the MJ Research PTC-2004 thermocylcer using the following regime: 50°C (2 min), 95°C (15 min), 40 cycles of 95°C (30 sec), 60°C (1 min), plate read and 50°C (5 min). The melting curve was determined using the following protocol: heat from 60°C to 90°C, by 0.1°C increments, holding for 5 seconds before reading the fluorescence of the samples.

Nested qPCR results were determined by purifying the PCR products using the QIAquick PCR Purification Kit, as per the manufacturer's protocol, and then running the purified samples through qPCR, as described above.

QA/QC Requirements. To determine if and where potential contamination or interference occurred during sample processing, positives and reagent blanks or negatives and matrix spikes of the PCR and qPCR samples were prepared. A positive control consisting of pure DNA (known to amplify by specific DNA primers) was used for the PCR and qPCR procedure. A matrix spike consisting of pure DNA (known to amplify by specific DNA primers) was used for the PCR and qPCR procedure. Negative controls consisted of water-only blanks for the PCR and qPCR procedure. The qPCR reactions were run in triplicate for each sample to determine the reproducibility of the method.

Results:

The samples arrived at the lab in good condition at 4°C with ice still in the cooler. The samples were filtered in the lab, and the filters were immediately placed in a -80°C freezer and stored until the DNA extraction was performed. Table 2 summarizes the qPCR analysis of the poultry project samples. The DNA extraction negative control and all PCR negative controls did not amplify any product. In addition, all calibration control checks were within acceptable values.

Table 2. Results of molecular analyses for the poultry samples.

Sample ID	Matrix	DNA (mg/L or mg/g)[a]	qPCR Poultry Specific Biomarker (copies/L water or g soil or g litter)[b]			qPCR Matrix Spike Amplified?[c]	Nested qPCR Amplified?[d]	Biomarker Melt Peak Identified?[d]	Other Melt Peaks Observed?
16861-7-20-06	Water	0[a]	BDL			Yes	No	N/A	N/A
BLFOSP-6-7-06	Water	4.4	Present			Yes	Yes	Yes	No
BS-HF22A-5-2-07	Water	0[a]	BDL			Yes	No	N/A	N/A
BS-HF22-SW-8-24-05	Water	1.0	BDL			Yes	No	N/A	N/A
ELMSPRQ-6-28-06	Water	0[a]	BDL			Yes	No	N/A	N/A
EOF-07-230-4-24-07	Water	6.4	BDL			Yes	No	N/A	N/A
EOF-07-232-4-24-07	Water	6.2	BDL			Yes	No	N/A	N/A
EOF-259-4-13-07	Water	55.9	BDL			Yes	No	N/A	N/A
EOF9-6-8-05	Water	16.8	BDL			Yes	No	N/A	N/A
EOF-Q1-6-17-06	Water	11.3	BDL			Yes	No	N/A	N/A
EOF-Q4-6-18-06	Water	1.5	BDL			Yes	No	N/A	N/A
EOF-SPRD-26-4-25-06	Water	89.5	1.81E+06	±	5.46E+05	Yes	N/A	Yes	No
EOF-SPREAD-025-5-4-06	Water	1,301	BDL			Yes	No	N/A	N/A
EOF-SPREAD-26-01-4-29-06	Water	102.6	BDL			Yes	No	N/A	N/A
EOF-SPREAD-59-01-4-29-06	Water	15.6	BDL			Yes	No	N/A	N/A
GPGW-20-6-11-30-06	Water	10.2	BDL			Yes	No	N/A	N/A
GPGW-48-7-12-1-06	Water	0.4	BDL			Yes	No	N/A	N/A
GW-Choats-01	Water	0[a]	BDL			Yes	No	N/A	N/A

GW-JONES-012311	Water	1.5	BDL			Yes	No	N/A	N/A
GW-McCOY-012212	Water	0 [a]	BDL			Yes	No	N/A	N/A
HESTENSP1-6-8-06	Water	21.5	BDL			Yes	No	N/A	N/A
HFS-02-5-10-06	Water	0 [a]	Present			Yes	Yes	Yes	No
HFS-02-INITIAL-4-29-06	Water	0 [a]	Present			Yes	Yes	Yes	No
HFS02Libby-6-15-05	Water	7.3	1.13E+05	±	4.65E+04	Yes	N/A	Yes	No
HFS04-BF1-01-6-15-06	Water	0.2	BDL			Yes	No	N/A	N/A
HFS05A-9-13-05	Water	3.4	BDL			Yes	No	N/A	N/A
HFS05-BF1-01-6-15-06	Water	0.3	Present			Yes	Yes	Yes	No
HFS05-BF1-7-12-05	Water	0 [a]	BDL			Yes	No	N/A	N/A
HFS14-BF2-01-8-1-06	Water	0.7	Present			Yes	Yes	Yes	Yes
HFS-14PEAK-4-7-06	Water	1.2	Present			Yes	Yes	Yes	No
HFS16-8-14-05	Water	24.4	Present			Yes	Yes	Yes	No
HFS16-9-15-05	Water	0.09	Present			Yes	Yes	Yes	No
HFS16-BF1-7-12-05	Water	4.8	BDL			Yes	No	N/A	N/A
HFS16-BF2-03-8-27-05	Water	4.5	6.61E+04	±	8.11E+03	Yes	N/A	Yes	No
HFS20-BF2--01-8-1-06	Water	2.4	BDL			Yes	No	N/A	N/A
HFS22-9-15-05	Water	3.192	BDL			Yes	No	N/A	N/A
HFS22A-6-15-05	Water	4.8	BDL			Yes	No	N/A	N/A
HFS22-BF1-01-6-15-06	Water	0.04	BDL			Yes	No	N/A	N/A
HFS22-BF2--01-8-1-06	Water	1.9	1.26E+05	±	5.76E+04	Yes	N/A	Yes	No
HFS23-7-16-05	Water	2.5	6.87E+03	±	2.96E+03	Yes	N/A	Yes	No
HFS23-7-23-05	Water	0.8	Present			Yes	Yes	Yes	No
HFS-28A-5-10-06	Water	0.5	Present			Yes	Yes	Yes	No
HFS-29PEAK-4-7-06	Water	4.8	BDL			Yes	No	N/A	N/A
LAL15SP2-7-11-06	Water	0.02	2.19E+03	±	5.94E+02	Yes	N/A	Yes	No
LAL16-GW1-7-18-06	Water	0 [a]	BDL			Yes	No	N/A	N/A

LIWSPR-6-28-06	Water	9.7	Present			Yes	Yes	Yes	Yes
LK-04-0-01-5-16-06	Water	3.5	BDL			Yes	No	N/A	N/A
LK04-0-01-9-26-06	Water	2.4	BDL			Yes	No	N/A	N/A
LOC-01-4-7-06	Water	21.7	BDL			Yes	No	N/A	N/A
Osborn-7-20-06	Water	0 [a]	BDL			Yes	No	N/A	N/A
RS-03-01-7-12-06	Water	4.4	Present			Yes	Yes	Yes	No
RS-109A-5-2-07	Water	0 [a]	Present			Yes	Yes	Yes	No
RS-122-050307A	Water	0.1	BDL			Yes	No	N/A	N/A
RS-127-BIO-8-11-06	Water	1.4	Present			Yes	Yes	Yes	Yes
RS-233-5-21-07	Water	158.8	3.29E+05	±	1.09E+05	Yes	N/A	Yes	No
RS-3-01-5-15-06	Water	20.0	BDL			Yes	No	N/A	N/A
RS-3-01-8-24-05	Water	1.2	BDL			Yes	No	N/A	N/A
RS-31-BIO-8-16-06	Water	0 [a]	BDL			Yes	No	N/A	N/A
RS-386A-5-2-07	Water	0 [a]	Present			Yes	Yes	Yes	Yes
RS-399A-5-2-07	Water	0.4	1.01E+04	±	2.88E+03	Yes	N/A	Yes	No
RS-433A-5-2-07	Water	0 [a]	BDL			Yes	No	N/A	N/A
RS-574-BIO-8-10-06	Water	8.4	BDL			Yes	No	N/A	N/A
RS-577-BIO-8-11-06	Water	2.4	Present			Yes	Yes	Yes	No
RS-578A-5-2-07	Water	0 [a]	Present			Yes	Yes	Yes	No
RS-625-BIO-8-10-06	Water	1.5	Present			Yes	Yes	Yes	No
RS-630-BIO-8-11-06	Water	8.8	BDL			Yes	No	N/A	N/A
RS-902-5-1-07	Water	0.9	Present			Yes	Yes	Yes	No
RS-BALLARD-5-5-06	Water	0.3	BDL			Yes	No	N/A	N/A
SP-JONES-012307	Water	0 [a]	BDL			Yes	No	N/A	N/A
Spread-52-4-25-06	Water	30.4	BDL			Yes	No	N/A	N/A
ZPEOF-001-4-25-06	Water	72.2	BDL			Yes	No	N/A	N/A
ZPEOF-30-4-25-06	Water	20.8	BDL			Yes	No	N/A	N/A

HFS08A-6-15-05	Water	0.51	Present			Yes	Yes	Yes	No
RS-122A-5-3-07	Water	0.13	BDL			Yes	No	N/A	N/A
Marth Guinn-7-25-06	Water	0 *	BDL			Yes	No	N/A	N/A
RS-3-01-9-25-06	Water	0 *	BDL			Yes	No	N/A	N/A
RS-340-BIO-8-15-06	Water	0.1	BDL			Yes	No	N/A	N/A
RS-43-BIO-8-10-06	Water	1.7	BDL			Yes	No	N/A	N/A

[a] "0" indicates that the DNA concentration was less than the detection limit.
[b] "Present" indicates that the biomarker was amplified, but was not quantifiable. "BDL" indicates below detection limits
[c] if "no" indicates that sample did not amplify with qPCR even after a sepharose cleanup was performed and the sample was diluted to a lower DNA concentration indicative of inhibition.
[d] N/A, not applicable. The sample was not run with the nested qPCR assay and/or the biomarker melt peak was not identified because the biomarker did not amplify in the qPCR sample run.

The poultry litter specific sample was detected in 38% of the samples and quantifiable in 11% of the samples. Non-specific amplification of organisms other than the target *Brevibacteria spp.* as evidenced by SYBR Green melt curves were identified in 4 samples: RS-386A-5-2-07, RS-127-BIO-8-11-06, LIWSPR-6-28-06 and HFS14-BF2-01-8-1-06.